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HATCHING OF *ILYANASSA OBSOLETA* EMBRYOS: DEGRADATION OF THE EGG CAPSULE PLUG IN THE ABSENCE OF DETECTABLE PROTEOLYSIS OF THE MAJOR PLUG PROTEINS

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ABSTRACT

The mechanism of action of the hatching substance released by *Ilyanassa obsoleta* embryos was examined by studying the sequential ultrastructural and biochemical changes that occur in the egg capsule plug as it is dissolved during hatching. When release of the hatching substance is triggered by incubating prehatching embryos in KCl, the inner two layers of the capsule wall (L3 and L4) that extend into the apex to form the plug separate from one another, but only in this region. The hatching substance then dissolves material at the periphery of the plug so that an intact plug can be recovered. However, if the plug is left in contact with the hatching substance, both the thin, electron dense material of the inner layer (L4) and the 10 nm filaments of the adjacent layer (L3) that compose most of the plug are dissolved. The first step in the hatching sequence is mimicked by papain so that L3 and L4 can be separated for analysis on SDS-polyacrylamide gels. L3 contains four major proteins with molecular weights of 24,000–52,000 daltons while L4 contains a predominant 25,000 dalton protein. When isolated plugs are dissolved in crude preparations of the hatching substance and analyzed by polyacrylamide gel electrophoresis, there is evidence of only slight disappearance of one minor plug protein. Based on these findings, *Ilyanassa* embryos probably release several activities necessary to dissolve the plug, yet degradation of the plug occurs without hydrolysis of the major plug proteins.

INTRODUCTION

Extracellular envelopes, which surround the eggs and embryos of nearly all animals, pose a barrier that must be penetrated at two critical periods of development. At fertilization, sperm must digest a passageway through these envelopes to successfully fuse with the egg cell surface. Accordingly, spermatozoa of many animals contain enzymes (McRorie and Williams, 1974; Hoshi *et al.*, 1979; Green and Summers, 1982; Yamada and Aketa, 1982) or lysins (Haino-Fukushima, 1974; Lewis *et al.*, 1982; Ogawa and Haino-Fukushima, 1984) within the acrosome that are released during fertilization. Development continues until the time of hatching when the envelopes must be penetrated again, this time by the embryo. Embryos escape from an envelope by one (or a combination) of three methods: an osmotic influx of water into an envelope to rupture it; mechanical movements of the embryo to break it; or the release of specific chemicals to weaken or dissolve it (reviewed by Davis, 1968). In most systems examined thus far, the chemicals released by embryos at hatching and those released by sperm during fertilization appear to be very similar to one another.

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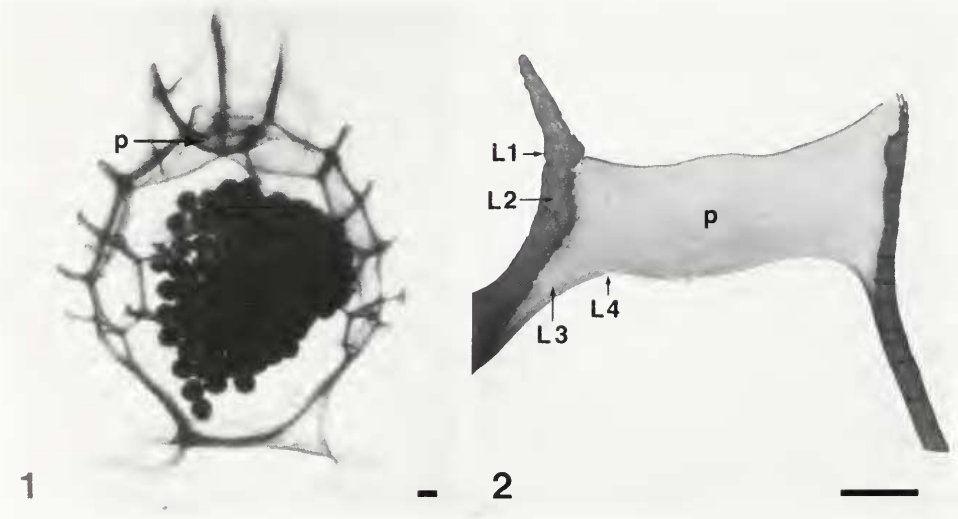
In these systems, sperm and embryos release divalent cation-requiring proteases, which is not surprising since envelopes are composed primarily of proteins (Katagiri, 1975; Bleil and Wassarman, 1980; Schoots and Denuce, 1981; Urch and Hedrick, 1981a).

The emergence of sea urchin and amphibian embryos from the fertilization envelope (a primary envelope produced by the oocyte) and fish embryos from a chorion (a secondary envelope derived from follicle cells) are the only systems in which the hatching process has been studied in detail. Considerable information is available on the hatching enzymes released by these embryos (see Sullivan and Bonar, 1984) yet the mechanism of action of these enzymes has been studied only during fish (Yamamoto and Yamagami, 1975; Iuchi and Yamagami, 1976; Yamagami, 1981) and frog (Yoshizaki, 1978; Urch and Hedrick, 1981b) hatching. Furthermore, similar data are not yet available on hatching from tertiary envelopes (secreted along the oviduct), so that it is impossible to draw any conclusions on the ubiquity of the hatching process for diverse animal groups.

One example of an oviduct-derived tertiary envelope is the multi-layered gastropod egg capsule. Of the few species of snails for which chemical hatching has been described, *Ilyanassa obsoleta* was chosen for our studies because adults reproduce readily and females deposit an abundance of egg capsules (Fig. 1) in the laboratory and because hatching is an early developmental event (Scheltema, 1967). After about a week of development embryos hatch as swimming veligers by releasing a hatching substance that dissolves the capsule plug, which occupies a small region of the egg capsule apex (Pechenik, 1975).

We have shown that the *Ilyanassa* hatching substance is a divalent cation-requiring protein that shares many of the biochemical characteristics of other well studied hatching enzymes (Sullivan and Bonar, 1984). It remains to be determined if the *Ilyanassa* hatching substance is also a protease. Along with biochemical studies on the hatching



FIGURES 1 AND 2. Light micrographs of the *Ilyanassa obsoleta* egg capsule. The plug (p) is at the apex of the capsule (Fig. 1). The location of the four layers of the egg capsule wall is revealed from a cross section through the apex (Fig. 2). The outer layer (L1) and the second layer (L2) form most of the capsule wall while the third layer (L3) expands in the apex to form most of the plug (p). The inner layer (L4) is not resolved by light microscopy, but its position lining the embryonic chamber is shown. Bar = 100 μm.

substance itself, we have undertaken an alternative approach to studying the function of this protein by examining its action on its natural substrate, the egg capsule.

In this study, we have identified three steps to the hatching process: (1) separation of the innermost capsule wall layer (L4) from its adjacent layer (L3) only in the plug region; (2) digestion of possible "cementing" material around the periphery of the plug; and (3) plug degradation itself. The first step of the hatching sequence can be mimicked by the protease papain, suggesting that the hatching substance has protease activity. However, the primary activity of the hatching substance, that of dissolving the plug, occurs in the absence of proteolysis of the major plug proteins, so that it is not clear whether the hatching substance is in fact a hatching enzyme.

Materials and Methods

Development of embryos

Ilyanassa adults, obtained from the Marine Biological Laboratory in Woods Hole, Massachusetts, or collected from mudflats near Lewes, Delaware, were maintained in aerated artificial seawater (Instant Ocean), 28‰ at 10°C. Groups of 35–40 snails were transferred to a 5 gallon aquarium at 23°C and fed frozen shrimp every other day to initiate reproduction. Egg capsule deposition began within a few days and capsules were collected daily from the aquarium walls. Capsules were rinsed briefly in 50% ethanol and cultured at 23°C in filtered seawater containing 20 μg/ml of the antibiotic rifampicin. Under these conditions, the majority of embryos hatch on day eight of development (Sullivan, 1984), yet it is difficult to predict when hatching from an individual capsule will occur. However, we have been able to experimentally initiate release of the hatching substance from embryos by briefly incubating embryos in a solution containing 280 m*M* KCl (60% 470 m*M* KCl and 40% K^+-free seawater, hereafter referred to as KCl) (Sullivan, 1983). With this technique, it has been possible to synchronize hatching from many capsules, required for the experiments outlined below.

Transmission electron microscopy

In order to observe the sequential changes in the capsule ultrastructure following release of the hatching substance, groups of 25 eight-day-old capsules were placed in a 10 ml dish of KCl to trigger release of the hatching substance. At five minute intervals, five capsules were removed and were immediately fixed in 5 m*M* 3-(N-Morpholino)propanesulfonic acid (MOPS) buffered seawater (pH 8.0) containing 2.5% glutaraldehyde. Capsules were cut in half below the plug and the apical region was postfixed in 1% aqueous OsO_4, dehydrated through an ethanol series, and embedded in Epon as described earlier (Sullivan and Maugel, 1984). Sections were cut with a diamond knife on a Sorvall MT-2B ultramicrotome, collected on copper grids, and stained with 2% aqueous uranyl acetate and 0.2% aqueous lead citrate (Venable and Coggeshall, 1965). Specimens were examined and photographed on a Hitachi HU-12 or an RCA EMU-3H transmission electron microscope.

Polarity of action of the hatching substance

Hatching enzymes normally digest inner portions of an envelope, then progressively digest outer layers. In sea urchin embryos, however, synchronous hatching occurs because the hatching enzyme released from older embryos can degrade the fertilization envelopes of younger embryos from the outside-in (Kopac, 1941). Therefore, we tested whether the *Ilyanassa* hatching substance could digest capsule plugs from the outside-

in as well as from the inside-out by placing intact capsules or opened capsules in preparations of crude hatching seawater prepared as described previously (Sullivan and Bonar, 1984). Twenty 8-day-old capsules were cut open into a solution of seawater and capsule fragments were removed. The embryos were then gently pelleted and seawater was withdrawn and replaced with a solution of KCl to induce release of the hatching substance. After 45 minutes, embryos were pelleted again and the resulting supernatant served as a crude preparation of the hatching substance. The predominant embryonic protein in this solution is a 55,000 dalton protein which may be the hatching substance. Before an experiment a 50 μl aliquot of each batch of the hatching substance was tested for its biological activity of removing a plug from the apex of a newly deposited capsule.

Incubation of egg capsules with enzymes

We attempted to mimic the action(s) of the hatching substance by monitoring the sensitivity of the capsule and plug to enzymatic digestion. One-day-old capsules were opened and the embryos and capsule fluid washed away. Capsule fragments were incubated in various commercial (Sigma) proteases (chymotrypsin, elastase, papain, pepsin, pronase, proteinase K, trypsin) or carbohydrases (snail gut enzymes, hyaluronidase, chitinase) at 1 mg/ml in an appropriate buffer for up to six hours and monitored for obvious changes in morphology.

Polyacrylamide gel electrophoresis

When isolated capsule plugs are homogenized in sample buffer (Laemmli, 1970), and examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), three glycoproteins are resolved having molecular weights of 49,000, 29,000, and 24,000 daltons (Sullivan and Maugel, 1984). In the initial analysis of plugs by SDS-polyacrylamide gel electrophoresis proteins were identified with Coomassie blue and the periodic acid-Schiff's stains. In the experiments below, we used the highly sensitive silver stain procedure (Oakley *et al.*, 1980) which allowed us to use fewer plugs per experiment while still detecting all three plug proteins in our samples. In one set of experiments plugs dissected from newly deposited capsules or plugs released from day eight capsules following KCl treatment were examined for similarities in protein profile to confirm that the proteins we identified were all from the plug and not part of the capsule wall. It was also possible to examine the proteins present in both regions of the plug (L3 and L4) because these layers could be separated by enzyme treatment. A final group of experiments was designed to determine if any (or all) of the plug proteins were sequentially degraded as hatching proceeded by placing isolated plugs in crude preparations of the hatching substance prepared as described above. Groups of twenty intact plugs were added to a 50 μl aliquot of the crude hatching substance for various times and digestion was stopped by the addition of an equal volume of 20% trichloroacetic acid (TCA) to precipitate proteins. Plugs that were soft and partially digested (see Fig. 4) were collected after six hours and completely digested plugs were collected at 12 and 24 hours. A similar series of digestions were performed on isolated pieces of L4. TCA precipitable material was pelleted at 10,000 $\times$ *g* and the pellet was resuspended in 1$\times$ Laemmli sample buffer, heated at 95°C for 2 minutes, and loaded onto a 12.5% polyacrylamide gel. Molecular weight markers (Pharmacia) were separated on each gel for determination of the molecular weights of plug proteins (Weber and Osborn, 1969).

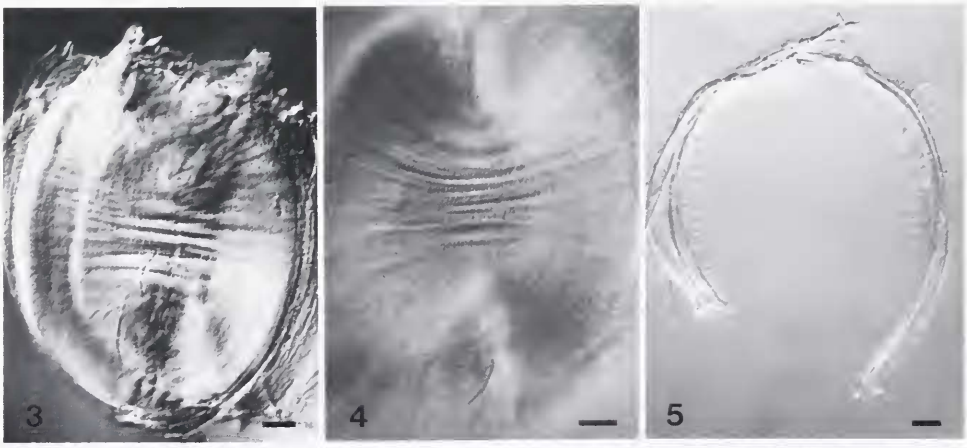
RESULTS

Changes in capsule morphology during hatching

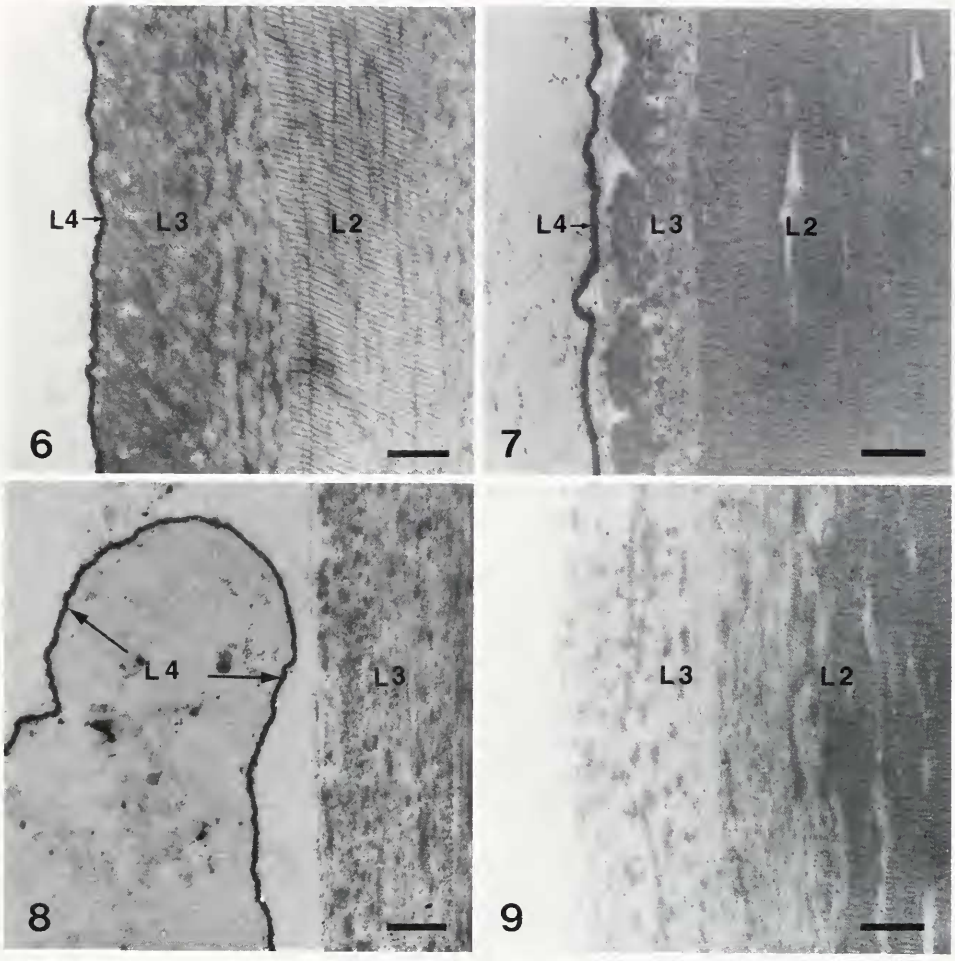
The *Ilyanassa* egg capsule wall contains four distinct layers that are most easily distinguished by their appearance in transmission electron micrographs (Sullivan and Maugel, 1984). The outer two layers of the capsule, L1 and L2, extend into the capsule apex and are adjacent to the plug but do not contribute material to the plug. The inner two layers of the wall, L3 and L4, are present throughout the capsule and extend into the apical region forming the plug, which is predominantly L3 (Fig. 2).

The capsule plug is a small disk 400–500 μm in diameter and 100–150 μm in thickness (Fig. 3). Plugs can be dissected manually from capsules, or will drop out intact from whole capsules within 10–15 minutes of KCl treatment. When plugs are exposed to the hatching substance for longer periods, they become soft and diffuse (Fig. 4). During natural hatching, plugs are quickly dissolved, but when isolated plugs are placed in crude preparations of the hatching substance, dissolution is slowed, probably because the hatching substance is less concentrated. Whereas a plug dissolves naturally in about an hour, isolated plugs take between 2–4 hours to dissolve in our experiments. Such plugs are completely dissolved, while portions of the adjacent capsule wall (probably L2) remain (Fig. 5).

When hatching is initiated by placing intact eight-day-old capusles in KCl, the various steps of the hatching process leading to plug removal can be observed. No changes in the capsule ultrastructure were noted within the first five minutes of KCl treatment. We believe the hatching subtance has been released, but has not had sufficient time to act. Capsule walls appeared indistinguishable from those of newly deposited capsules (Sullivan and Maugel, 1984). The striated fibers of L2, the filaments of L3, and the electron dense layer of L4 remained intact and closely apposed (Fig. 6). After 10 minutes, the hatching substance has caused L4 to separate from L3 (Figs. 7, 8). Although L4 appears uniform throughout the egg capsule (Sullivan and Maugel,



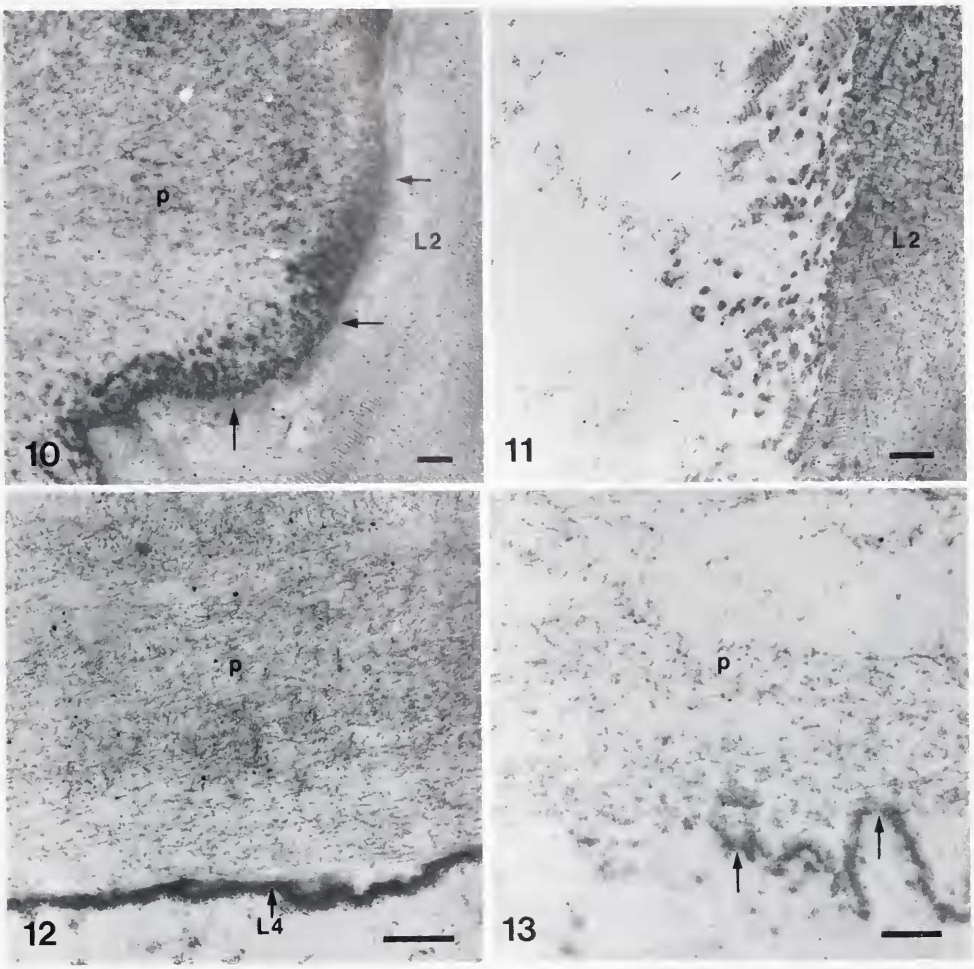
FIGURES 3–5. Nomarski interference contrast light micrographs of the digestion of an egg capsule plug. Prior to day eight, plugs remain firmly in place in the capsule apex but can be dissected from this area (Fig. 3). When a capsule plug is added to the hatching substance, plug filaments become very diffuse within 60 minutes (Fig. 4). With prolonged incubation in the hatching substance, the plug has dissolved, while material from the adjacent layer remains undigested (Fig. 5). Bar = 50 μm.



FIGURES 6–9. Transmission electron micrographs of *Ilyanassa* egg capsules incubated in KCl to initiate release of the hatching substance. After five minutes of KCl treatment, no changes are visible in the capsule wall (L2–L4 are shown with the inside of the capsule to the left) and all layers remain in close contact (Fig. 6). By 10 minutes L4 has begun to separate from L3 slightly below the apex (Fig. 7) but has pulled away considerably in the plug region (Fig. 8). After 15 minutes of treatment, the first phase of hatching is completed when L4 has been completely removed from the apex and the hatching substance has access to the filaments of L3 (Fig. 9). Bar = 0.5 μm.

1984), it separates from L3 and dissolves only in the capsule apex (Fig. 9). In other regions of the capsule, L4 remains tightly adherent to L3.

Around this time, L4 begins to dissolve and the hatching substance now has access to inner regions of the capsule apex. The next change in the capsule structure occurs at the margins of the plug where it contacts L2. The area of close contact between the plug and the fibers of L2 (Fig. 10) begins to loosen, perhaps as a result of the hatching substance digesting material holding the plug in place. The hatching substance apparently works very quickly because within 15 minutes of KCl treatment, material present between the plug and L2 (Fig. 10) is no longer present (Fig. 11) and intact plugs can be recovered from about 50% of the capsules tested (Sullivan, 1983).



FIGURES 10–13. Transmission electron micrographs of the second and third phases of the hatching process. During the second phase the area of close contact between L2 and the plug (p) is disrupted, possibly by the digestion of material between these layers (arrows) that may hold the plug in place (Figs. 10, 11). During the final step of the hatching process the two layers of the plug (L3 and L4) that remain intact during the first 15 minutes of KCl treatment (Fig. 12) are quickly digested by the hatching substance (Fig. 13). Bar = 0.4 μm.

If a capsule plug is left undisturbed, both the 10 nm filaments of L3 and the 60 nm wide electron dense material of the L4 are quickly dissolved (Figs. 12, 13), so that after an hour the plug is gone. Therefore, hatching is divided into three phases: separation of L4 from L3; degradation of material at the periphery of the plug so that a plug can be recovered intact; and dissolution of the plug itself.

Polarity of the hatching substance

When intact capsules were incubated in crude preparations of the hatching substance so that only the external plug surface was exposed, plugs were never removed.

Control capsule apices cut open to expose the inner side of the plug to the hatching substance had their plugs dissolved in all replicates of this experiment.

Protease and carbohydrase action on the capsule

Attempts to mimic the action of the hatching substance with commercial proteases or carbohydrases were unsuccessful with one exception. Incubation of capsule apices in papain (10 mg/ml in 10 m*M* Tris pH 7.6 diluted 1:1 with filtered seawater) loosened the contact between L4 and L3 so that L4 could be separated easily from the rest of the capsule (Fig. 14). With this 0.5% solution of papain, L4 began to separate from L3 in less than 60 seconds, with less concentrated papain solutions taking longer.

Polyacrylamide gel electrophoresis

Silver stained gels of *Ilyanassa* egg capsule plugs had protein profiles that differed somewhat from our earlier results (Sullivan and Maugel, 1984). The 49,000 dalton band was resolved into a doublet of 52,000 and 48,000 daltons and additional proteins with molecular weights in excess of 95,000 daltons were detected (Fig. 15a).

In our previous work, whole plugs were dissected from capsule apices. It was possible that some of the proteins resolved on gels were part of L2 adhering to the plug (see Fig. 5). Therefore, we treated capsules with KCl to separate plugs from the wall prior to analyzing plug proteins. It was also possible to determine whether any of the plug proteins identified previously were from L4 by removing this layer from the remainder of the plug with papain. With these additional two treatments, identical protein profiles were observed (Figs. 15b, c) indicating that the four proteins we have identified were in fact from the plug, and furthermore, all were from L3.

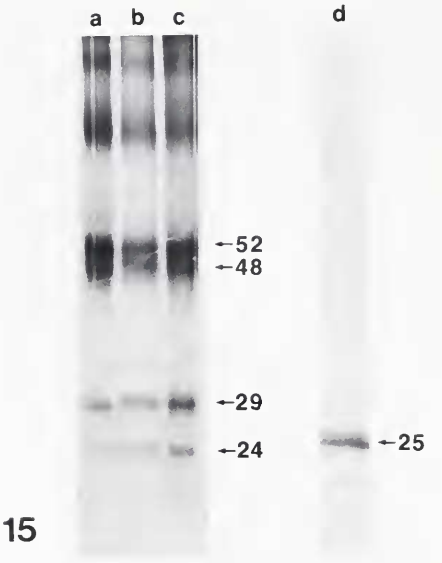
Fragments of L4 that were separated from L3 with papain were also analyzed by gel electrophoresis. L4 contained a prominent 25,000 dalton protein (Fig. 15d) and occasionally a faint 15,000 dalton protein was detected (see Fig. 17).

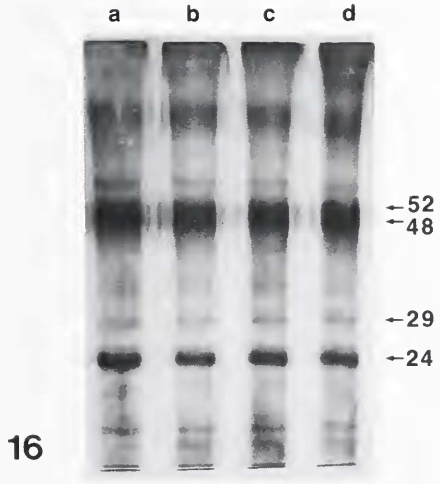
Plugs that have been in contact with the crude hatching substance for about six hours and are partially dissolved, (see Fig. 4) still contain the same four proteins seen in plugs from newly deposited capsules (Fig. 16). These four proteins persist intact for up to 24 hours even when digestion has gone to completion and plugs are no longer visible. We also analyzed samples of L4 for evidence of proteolysis by the hatching substance. There is only minor reduction of the 25,000 dalton protein by 24 hours, well after this layer has been digested by the hatching substance (Fig. 17).

Discussion

Although hatching from egg envelopes is a ubiquitous problem for embryos, what is known about chemical hatching has come from studies of very few species whose embryos emerge from primary or secondary envelopes. We have selected *Ilyanassa* as an example of embryos enclosed within a tertiary envelope to compare its hatching mechanism to what is known about the hatching processes and hatching enzymes of the other more thoroughly studied embryos.

Much of the early work on the characterization of hatching enzymes utilized artificial protein substrates which generally are not degraded very quickly, suggesting that the enzymes have high specificity for their natural substrates, which are degraded very rapidly (Ishida, 1944; Kaighn, 1964). The preferred approach now is to analyze the natural substrate of a hatching enzyme directly (Yamagami, 1970; 1973). For example, products released from radioactively labeled envelopes can be quantitated under a variety of conditions (pH, temperature, ions, protease inhibitors) to study the





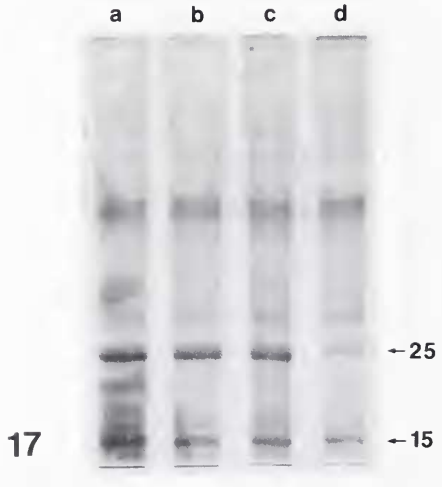


FIGURE 14. Light micrograph of a capsule apex treated with 0.5% papain. The innermost layer (L4) quickly separates from the rest of the capsule wall, but the plug (p) remains in place. Bar = 50 μm.

FIGURE 15. Analysis of *Ilyanassa* capsule plug proteins on a 12.5% polyacrylamide gel. Plugs were dissected from newly deposited capsules (a), were released from 8-day capsules following incubation in KCl for 20 minutes (b), or were incubated in papain to remove L4 then dissected from capsules (c). Pieces of L4 were also analyzed (d). Molecular weights (in kdaltons) of the major proteins are indicated.

FIGURES 16–17. Analysis of digestion of capsule plug proteins on a 12.5% polyacrylamide gel. Entire plugs (Fig. 16) or pieces of L4 alone (Fig. 17) were incubated in the hatching substance for 0, 6, 12, and 24 hours (lanes a–d) then prepared as described. Locations of the major proteins (in kdaltons) are indicated. The high background staining on these gels is due to proteins present in the crude hatching substance.

activity of a hatching enzyme (DiMichelle *et al.*, 1981; Urch and Hedrick, 1981a). Another useful approach is to examine sequential biochemical or ultrastructural changes in an envelope as a consequence of the release of a hatching enzyme (Yamamoto and Yamagami, 1975; Iuchi and Yamagami, 1976; Yoshizaki, 1978; Urch and Hedrick, 1981b). We have utilized these latter approaches in our analysis of the *Ilyanassa* hatching substance to determine how it functions and if it may be a protease.

Our results support an earlier conclusion that the onset of hatching is rapid and is controlled by the sudden release of the hatching substance (Sullivan, 1983). There is no evidence from transmission electron micrographs that any layer of the capsule wall or plug is altered prior to day eight, nor do the plug proteins undergo any gradual degradation during the prehatching period. Hatching is also a very sudden event for some fish embryos with the rapid digestion of the *Oryzias latipes* chorion (Yamamoto and Yamagami, 1975) being accompanied by the release of at least six soluble glycoproteins (Iuchi and Yamagami, 1976). However, two high molecular weight proteins of the vitelline envelope around *Rana japonica* embryos are slowly degraded during the two to three days preceeding hatching (Yoshizaki, 1978). Therefore, hatching is a gradual process for the embryos of this frog.

There are two other features of the hatching mechanism utilized by embryos that we have examined for *Ilyanassa.* As mentioned above, some hatching enzymes degrade an envelope equally well from either side. This is not the case for *Ilyanassa.* Although the hatching substance can degrade all parts of the egg capsule plug, it apparently must do it in the proper sequence. Another difference is that while the envelopes of fish, frog, and sea urchin embryos are entirely dissolved during hatching, almost all of the *Ilyanassa* egg capsule persists after hatching. It is not clear why L4, which lines the entire embryonic chamber (Sullivan and Maugel, 1984), is only digested in the capsule apex. Removal of L4 from the apex gives the hatching substance access to the L3 portion of the capsule plug leading to plug digestion, yet L3 also remains intact throughout the rest of the capsule. The outer two layers of the capsule wall also remain intact so that much of the capsule of *Ilyanassa,* like those of other marine gastropods, is resistant to hatching "enzymes" (Fretter, 1941). This finding is best explained by differences in amino acid composition of capsule plugs and the outer layers of the capsule wall. For example, amino acid analysis of the *Busycon* capsule walls failed to detect cysteine or tryosine residues, while both of these amino acids were detected in protein hydrolyzates of plugs (Harasewych, 1978), leading to the conclusion that the *Busycon* hatching enzyme must have chymotrypsin-like specificity. Similarly, histochemical tests for specific amino acids revealed that stains for tyrosine and tryptophan residues were strongly positive in the plug of the *Nucella* capsule while the capsule wall was deficient in these amino acids (Bayne, 1968).

We have implied that the hatching substance is a single functional protein with one activity, but this probably is not the case. Results from the ultrastructural analysis of hatching suggest that there could be three or four separate activities released by embryos that are needed to separate L4 from L3, to digest material at the plug-wall junction, and to dissolve L4 and L3. Therefore, it is not a simple matter to assign a mechanism to what may be a very heterogeneous hatching substance. Because of the protein composition of the capsule plug, it is tempting to speculate that the hatching substance would be a protease, but the plug and entire capsule are very resistant to digestion by almost all of the proteases tested. One of the early events of hatching is mimicked by papain, so perhaps the hatching substance has a proteolytic component, but the primary activity of dissolving the plug is occurring by very limited proteolysis of the plug. The four major proteins of L3 that compose virtually all of the plug have identical molecular weights on polyacrylamide gels when we analyze samples of plugs

that are either intact, partially digested, or completely dissolved. Thus the major activity of the hatching substance is not as a protease that degrades these four proteins. It is possible that the major plug proteins are held together by lower molecular weight cross-linking proteins and it is these cross-linking proteins that are the substrate of the hatching substance. We have not detected any such proteins when plugs were examined on 20% polyacrylamide gels, suggesting that if such cross-linking proteins exist, they would have molecular weights less than 8–10,000 daltons.

An alternative explanation for the action of the hatching substance is that it is functioning as a lysin. The primary criterion for distinguishing a lysin from an enzyme is how it interacts with its substrate: either catalytically to release distinct products (an enzyme), or stoichiometrically, remaining bound and not releasing distinct products (a lysin) (Lewis *et al.,* 1982). Two observations suggest the hatching substance of *Ilyanassa* may be working in a manner consistent with it being a lysin. First, the four plug proteins from L3 were not degraded by the hatching substance when analyzed on SDS-polyacrylamide gels. Similarly, there is no change in the migration of the major vitelline envelope proteins before and after prolonged incubation with the *Haliotis* sperm lysin (Lewis *et al.,* 1982). Second, it appears that the plug dissolving activity of crude preparations of the hatching substance can be saturated when additional plugs are added. In our assays of hatching activity, a single plug is usually dissolved in two or three hours (Sullivan and Bonar, 1984). However, when additional plugs are added to separate aliquots of the same batch of the hatching substance, plug digestion no longer occurs (Sullivan, unpub.). A true enzyme would be expected to dissociate from its substrate and be available to work on additional plugs, but a lysin remains bound to its substrate.

We have detected low levels of protease activity released at hatching (Sullivan and Bonar, 1984) that may correspond to the papain-like activity that separates L4 from L3. Therefore, there may be a proteolytic component to the hatching substance. However, the primary activity of the hatching substance is not one that hydrolyzes the major plug proteins. These results provide valuable information on how we should approach the characterization of the hatching substance of *Ilyanassa* in our future experiments.

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LITERATURE CITED

BAYNE, C. J. 1968. Histochemical studies of the egg capsule of eight gastropod molluscs. *Proc. Mallacol. Soc. Lond.* **38:** 199–212.

BLEIL, J. D., AND P. M. WASSARMAN. 1980. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* **76:** 185–202.

DAVIS, C. C. 1968. Mechanisms of hatching in aquatic invertebrate eggs. *Oceanog. Mar. Biol. Ann. Rev.* **6:** 325–376.

DIMICHELE, L., M. H. TAYLOR, AND R. SINGLETON, JR. 1981. The hatching enzyme of *Fundulus heteroclitus*. *J. Exp. Zool.* **216:** 133–140.

FRETTER, V. 1941. The genital ducts of some British stenoglossan prosobranchs. *J. Mar. Biol. Assoc. U. K.* **25:** 173–211.

GREEN, J. D., AND R. G. SUMMERS. 1982. Effects of protease inhibitors on sperm-related events in sea urchin fertilization. *Dev. Biol.* **93:** 139–144.

HAINO-FUKUSHIMA, K. 1974. Studies on the egg membrane lysin of *Tegula pfeifferi:* the reaction mechanism of the lysin. *Biochim. Biophys. Acta* **352:** 179–191.

HARASEWYCH, M. G. 1978. Biochemical Studies of the Hatching Process in *Busycon.* Master's Thesis, University of Delaware, Newark. 52 pp.

HOSHI, M., T. MORIYA, T., AOYAGI, AND H. UMEZAWA. 1979. Biochemical studies of the hatching process in sea urchin embryos. I. Effects of protease inhibitors. *J. Exp. Zool.* **209:** 129–134.

ISHIDA, J. 1944. Further studies on the hatching enzyme of the freshwater fish, *Oryzias latipes. Ann. Zool. Japn.* **22:** 155–164.

IUCHI, I., AND K. YAMAGAMI. 1976. Major glycoproteins solubilized from the teleostean egg membrane by the action of the hatching enzyme. *Biochim. Biophys. Acta* **453:** 240–249.

KAIGHN, M. E. 1964. A biochemical study of the hatching process in *Fundulus heteroclitus. Dev. Biol.* **9:** 56–80.

KATAGIRI, C. 1975. Properties of the hatching enzyme from frog embryos. *J. Exp. Zool.* **193:** 109–118.

KOPAC, M. J. 1941. Disintegration of the fertilization membrane of *Arabacia* by the action of an "enzyme." *J. Cell. Comp. Physiol.* **18:** 215–220.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

LEWIS, C. A., C. F. TALBOT, AND V. D. VACQUIER. 1982. A protein from abalone sperm dissolves the egg vitelline layer by a nonenzymatic mechanism. *Dev. Biol.* **92:** 227–239.

MCRORIE, R. A., AND W. L. WILLIAMS. 1974. Biochemistry of mammalian fertilization. Pp. 777–801 in *Annual Review of Biochemistry,* Vol. 43, E. E. Snell, ed. Annual Reviews, Inc., Palo Alto.

OAKLEY, B. R., D. R. KIRSCH, AND N. R. MORRIS. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105:** 361–363.

OGAWA, A., AND K. HAINO-FUKUSHIMA. 1984. Isolation and purification of vitelline-coat lysin from testis of *Turbo cornutus* (Mollusca). *Dev. Growth Differ.* **26:** 345–360.

PECHENIK, J. A. 1975. The escape of veligers from the egg capsules of *Nassarius obsoletus* and *Nassarius trivittatus* (Gastropoda, Prosobranchia). *Biol. Bull.* **149:** 580–589.

SCHELTEMA, R. S. 1967. The relationship of temperature to the larval development of *Nassarius obsoletus* (Gastropoda). *Biol. Bull.* **132:** 253–265.

SCHOOTS, A. F. M., AND J. M. DENUCE. 1981. Purification and characterization of hatching enzyme of the pike *Esox lucius. Int. J. Biochem.* **13:** 591–602.

SULLIVAN, C. H. 1983. Precocious hatching of *Ilyanassa obsoleta* embryos exposed to $CaCl_2$ or KCl. *J. Exp. Zool.* **228:** 145–150.

SULLIVAN, C. H. 1984. Developmental alterations in the appearance of hatching activity in *Ilyanassa obsoleta* embryos. *Wilhelm Roux's Arch.* **193:** 219–225.

SULLIVAN, C. H., AND D. B. BONAR. 1984. Biochemical characterization of the hatching process of *Ilyanassa obsoleta. J. Exp. Zool.* **229:** 223–234.

SULLIVAN, C. H., AND T. K. MAUGEL. 1984. Formation, organization, and composition of the egg capsule of the marine gastropod, *Ilyanassa obsoleta. Biol. Bull.* **167:** 378–389.

URCH, U. A., AND J. L. HEDRICK. 1981a. Isolation and characterization of the hatching enzyme from the amphibian, *Xenopus laevis. Arch. Biochim. Biophys.* **206:** 424–431.

URCH, U. A., AND J. L. HEDRICK. 1981b. The hatching enzyme from *Xenopus laevis:* limited proteolysis of the fertilization envelope. *J. Supramolec. Struc. Cell. Biochem.* **15:** 111–117.

VENABLE, J. H., AND R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell. Biol.* **25:**407–408.

WEBER, K., AND M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.

YAMADA, Y., AND K. AKETA. 1982. Purification and characterization of trypsin like enzymes of sperm of the sea urchin *Hemicentrotus pulcherrimus. Dev. Growth Differ.* **24:** 125–134.

YAMAGAMI, K. 1970. A method for rapid and quantitative determination of the hatching enzyme (chorionase) activity of the medaka, *Oryzias latipes. Ann. Zool. Jpn.* **43:** 1–9.

YAMAGAMI, K. 1973. Some enzymological properties of a hatching enzyme (chorionase) isolated from the freshwater teleost, *Oryzias latipes. Comp. Biochem. Physiol.* **46B:** 603–616.

YAMAGAMI, K. 1981. Mechanisms of hatching in fish: secretion of hatching enzyme and enzymatic choriolysis. *Am. Zool.* **21:** 459–471.

YAMAMOTO, M., AND K. YAMAGAMI. 1975. Electron microscopic studies on choriolysis by the hatching enzyme of the teleost, *Oryzias latipes. Dev. Biol.* **43:** 313–321.

YOSHIZAKI, N. 1978. Disintegration of the vitelline coat during the hatching process in the frog. *J. Exp. Zool.* **203:** 127–134.